

POLLEN DEVELOPMENT IN *TRITICUM DURUM* DESF.: A HISTOCHEMICAL STUDY

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ABSTRACT

Pollen formation in the anther of *Triticum durum* Desf. was studied histochemically using Carnoy's fixed material and light microscopy. The substances localized were: DNA, RNA, proteins and ascorbic acid (AA) in the tissue sections and the content determined in different tissues.

UITTREKSEL

STUIFMEEL ONTWIKKELING IN *TRITICUM DURUM* DESF.: 'N HISTOCHEMIESE ONDERSOEK

Stuifmeel vorming in die helmknop van *Triticum durum* Desf. is histochemies met Carnoy-gefikseerde materiaal en ligmikroskopie ondersoek. Die stowwe gelokaliseer was DNA, RNA, proteïne en askorbinesuur (AA) in die weefselsnitte en die inhoud is vir verskillende weefsels bepaal.

INTRODUCTION

Although detailed morphological and cytological studies have been made on many important flowering plants, histochemical and physiological studies have been limited. Such studies, revealing the functions of different tissues of the anther, are now receiving considerable attention. In the present work an attempt has been made to substantiate the histochemical differentiation in the anther of *Triticum durum* Desf. during its successive ontogenetic stages. For this purpose, qualitative assessments of DNA, RNA, proteins and ascorbic acid were made employing standard histochemical methods on tissue sections in conjunction with light microscopy.

MATERIAL AND METHODS

Flower buds of *Triticum durum* Desf. fixed in Carnoy's medium were dehydrated in an ethanol-*n*-butanol series and embedded in paraffin. Serial microtome sections of 8 micron thickness were cut and mounted with gelatin adhesive.

RNA and DNA were localised using the azure B and Feulgen methods, respectively (Jensen, 1962). The following controls were run: RNA and DNA

were extracted with 5 % hot perchloric acid for 30 min at 60 °C (Erickson, 1949) (Fig. 14); RNA alone was extracted with 10 % cold perchloric acid (Kasten, 1965) (Fig. 15). Total proteins were demonstrated using the mercuric bromophenol blue (HgBPB) reaction (Mazia *et al.*, 1953).

In order to localise ascorbic acid (AA) the following silver nitrate (AgNO_3) method was adopted (Dave *et al.*, 1968): fresh flower buds were immersed in a solution containing 34 ml 5 % silver nitrate plus 66 ml 100 % alcohol and 5 ml acetic acid and left for one week in the refrigerator. Later, the treated material was thoroughly washed in 50 % ethanol and dehydrated as mentioned above. The paraffin sections were dried on the slides, deparaffinized in xylene and sealed with Canada balsam. Observations were made under the light microscope.

RESULTS

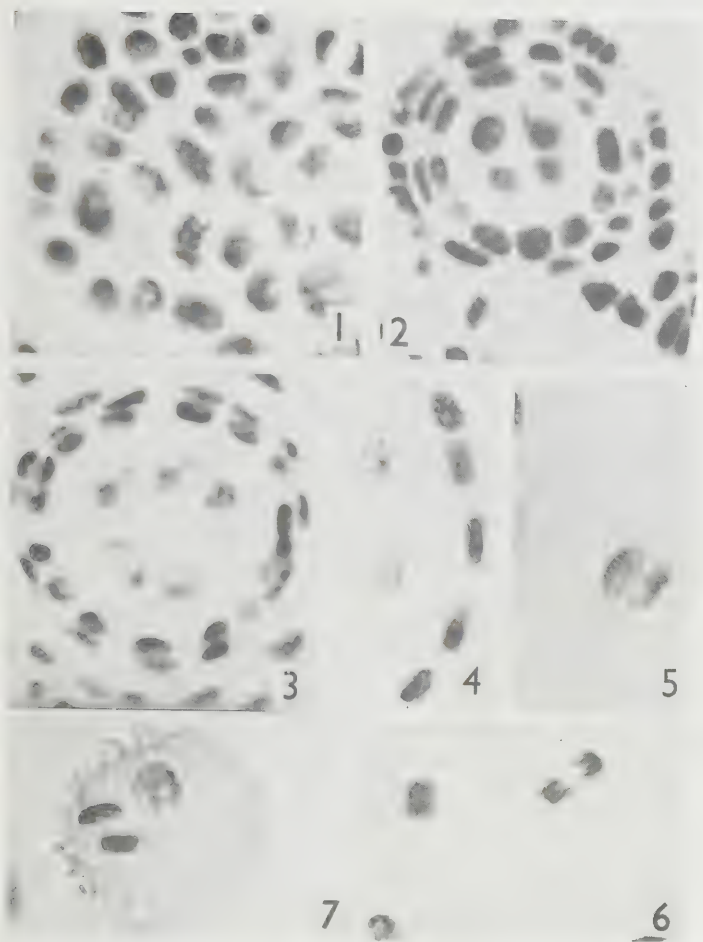
DNA: the hypodermal archesporial cell in the anther primordium appears to be rich in DNA, similar to the neighbouring cells (Fig. 1). In the early stages, the sporogenous tissue appears to be rich in DNA (Fig. 2) which decreases to a low level in the late stage (Fig. 3) and in the pollen mother cells. During meiosis-I a considerable increase in DNA content occurs at prophase. Although DNA is high at late anaphase-I, the resulting dyads and tetrads show low DNA.

After the separation of spores, the microspores in the resting phase contain low DNA (Fig. 4), while prior to mitosis, DNA again increases. Equal concentration of DNA occurs in both generative and vegetative cells (Figs 5, 6). Later, in the 2-celled pollen, DNA content in the generative cell increases again. In the mature 3-celled pollen, the male cells contain higher levels of DNA than the vegetative nucleus (Fig. 7).

In the course of entire anther development, the epidermal layer, endothecium and wall layers are rich in DNA (Figs 2, 3). When the PMC's are formed, the innermost wall layer functions as tapetum; its DNA content appears to increase to a high level (Figs 3, 4). This higher DNA content is maintained until it degenerates. In *Triticum*, binucleate tapetum is observed.

RNA: in the anther primordia, the archesporial cell is large and is rich in RNA (Fig. 8). The primary sporogenous tissue which is derived from the archesporium also retains a high concentration of RNA (Fig. 9). Later, the RNA level declines (Fig. 10) until ultimately, its level in the pollen mother cells is fairly low (Fig. 11). The nucleoli in all these phases are rich in RNA. The low cytoplasmic RNA level of the PMC's is continued during meiosis in the dyads and tetrads. The tetrads of spores are vacuolated; their nucleoli are rich in RNA (Fig. 12). During interphase an increase in RNA synthesis is observed; it is continued in the vegetative and generative cells of 2-celled pollen (Fig. 13).

Simultaneously with the differentiation of the sporogenous tissue, the primary parietal layer, which is rich in RNA, contributes to the endothecium. In the older anther, the endothecium and the epidermis, both of which were rich in RNA, show



FIGS. 1-7.

Anther sections of *Triticum durum* stained for DNA. Figs 1-3. Pre-meiotic stages showing high DNA levels in all the tissues, but low in the late sporogenous tissue in Fig. 3, X 400. Fig. 4. Post-meiotic stage. Note low intensity of DNA in the nuclei of microspores when compared to tapetum, X 600. Figs 5, 6. Mitotic divisions in the pollen grain showing relatively high DNA levels in both generative and vegetative cells, X 400. Fig. 7. 3-celled pollen, with the male cells, rich in DNA X 500.

a gradual fall in RNA content (Figs 10, 11). Similarly, the connective tissue also shows very low RNA. The tapetum synthesises RNA even at the connective side (Figs 10–12). In the mature anther, the endothelial layer develops wall thickenings which stain faintly green with azure B. Subsequently, the tapetum degenerates at the time of pollen formation, while maintaining its high RNA content.

PROTEINS: the young anther primordium is rich in proteins. Following the first parietal division in the archesporium the protein content of the nuclei of sporogenous cells is also high (Fig. 16). In the older anther, the sporogenous tissue is rich in cytoplasmic and nuclear proteins. During its further growth and differentiation into pollen mother cells, a sharp rise in protein content is observed (Fig. 17). The nucleoli also stain intensely for proteins. During meiosis-I, however, the cytoplasmic protein concentration seems to decline, while the proteins of spindles and chromosomes remain high (Figs 18, 19). This low cytoplasmic protein level is maintained during meiosis-II. At the time of microspore formation, the protein level increases again (Fig. 20). In the shedding pollen however, the male cells and the vegetative nucleus stain more intensely for protein than the cytoplasm (Fig. 21).

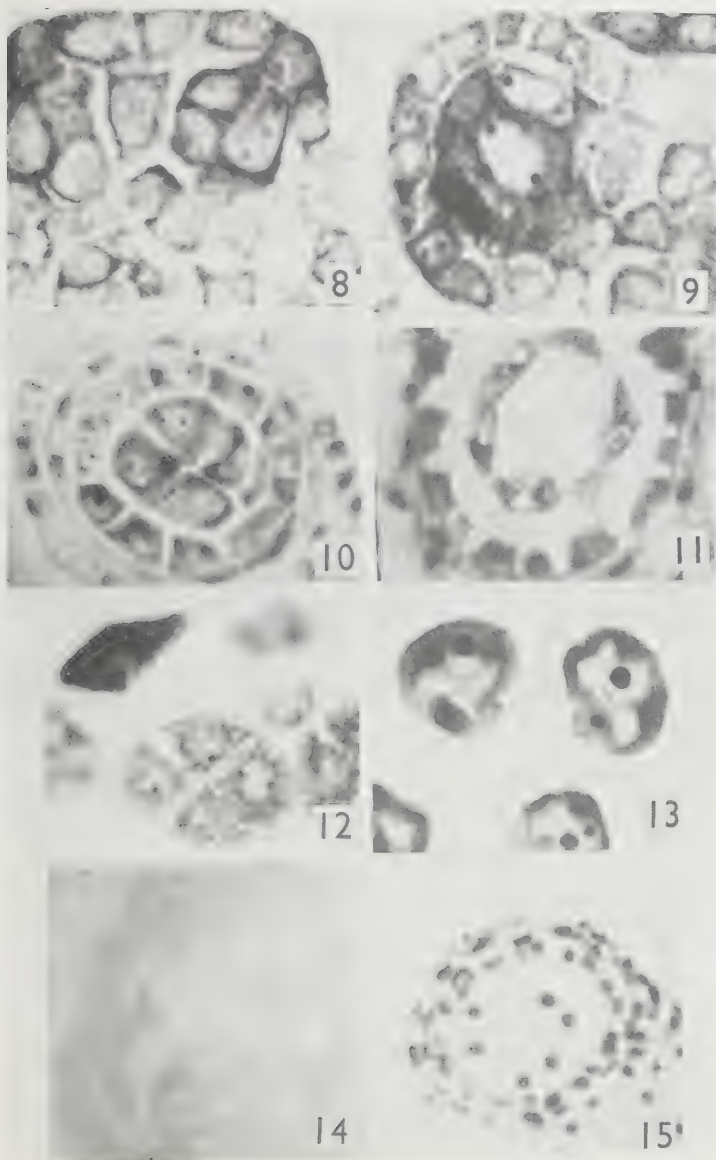
In the anther, as the wall layers differentiate, a decrease in protein content similar to that of RNA is observed. The reduction in the protein level occurs in the wall layers, centripetally with the successive differentiation of the latter. The epidermis also undergoes a decline in protein content. The glandular tapetum maintains a high concentration of proteins at all stages of anther growth (Figs 17–19).

ASCORBIC ACID (AA): Ascorbic acid is present in all the tissues of the anther. The young anther primordium stains uniformly for AA. No conspicuous distribution pattern of AA appears. However, in young sporogenous cells where only one parietal layer is differentiated, more AA appears in the latter (Fig. 22). The sporogenous tissue contains little AA; later, this tissue and tapetum appears to be richer in AA (Fig. 23), whereas the anther wall layers have a slightly lower AA content. In pollen mother cells a slight increase in AA synthesis occurs (Fig. 24). It appears to be more intense at the periphery of the cell walls and the nuclear membranes (Fig. 24). The same concentration is observed even at prophase and metaphase of the PMC's; the chromosomes stain a strong black. No additional synthesis takes place afterwards. At the tetrad stage, the spores show a peripheral

FIGS. 8–15.

Anther sections of *Triticum durum* stained for RNA. Figs 8–11. Pre-meiotic stages showing high RNA content in the sporogenous tissue and tapetum; however, it declines in pollen mother cells at the onset of meiosis (Fig. 11). Note the gradual reduction of RNA in the anther wall layers, X 600; Fig. 11, X 500. Fig. 12. Microspore tetrad and tapetum respectively showing low and high RNA, X 700. Fig. 13. 2-celled pollen high in RNA in the generative and vegetative cells, X 900. Figs 14, 15. Control tests for RNA and DNA.

Note both RNA and DNA (Fig. 14) and RNA alone extracted (Fig. 15), X 500.



deposition of AA (Fig. 25). The tapetum during meiosis resembles the PMC's in AA content, although its inner peripheral sheath contains more AA. The quantity and the mode of distribution of AA in PMC's is maintained in young microspores (Fig. 26). In 2-celled pollen, both vegetative and generative cells are rich in AA. The exine and intine are negative to the AA test. Mature pollen reacts similarly to 2-celled pollen. During meiosis the AA level in the anther wall layers is low; this is continued even at maturity.

DISCUSSION

In general, correlation of growth patterns of different anther tissues appears to be simple when viewed morphologically, but not so when observed histochemically. Normally, the early growth of the anther primordia is dominated by cell division until the differentiation of the various tissues. Thus, the archesporium and sporogenous cells enter the phase of specialized cell growth and differentiation which culminates in the formation of pollen. During this period the tapetum acts as an accessory (nursing) tissue. The remaining tissues of the anther show a progressive decline in cell division and differentiate mainly in terms of cell expansion.

During early growth of the anther, the amount of DNA, as indicated by its staining intensity in the sporogenous tissue gradually declines; it increases again at the prophase of meiosis. The relatively low DNA content of the resulting dyads, tetrads and young microspores as compared to that of the tapetum and anther wall layers correlates with the fact that the latter tissues are diploid and the former are haploid. This, however, has not been the case with the diploid sporogenous nuclei, where a low DNA content appears to be a feature (Rudramuniyappa, 1973; Panchaksharappa and Rudramuniyappa, 1974, 1975). It should be kept in mind, however, that low DNA stainability in the nuclei may be due to interference by basic proteins, the histones (Heslop-Harrison, 1972). The histones probably mask staining of DNA. If this possibility is correct, the neighbouring maternal tissues of

Figs. 16–26.

Anther sections of *Triticum durum* stained for proteins (Figs 16–21) and ascorbic acid (AA) (Figs 22–26). Figs 16, 17. Pre-meiotic stages showing high protein levels in the early sporogenous tissue, pollen mother cells and tapetum; note intense staining in the nucleoli, X600. Figs 18, 19. Meiosis. Note gradual reduction of proteins in the cytoplasm, but chromosomes and spindles are rich in proteins. Tapetum remains rich, but the wall layers are low in proteins, X 700. Figs 20, 21. One- and three-celled pollen respectively showing high protein levels in the cytoplasm, male cells and vegetative nucleus. Fig. 20, X 600; Fig. 21, X 800. Figs 22, 23. Pre-meiotic stages. Note the gradual synthesis of AA in sporogenous tissue and in newly differentiated tapetum (Fig. 23), X 700. Fig. 24. Pollen mother cells and tapetum synthesize AA; its location is mainly at the periphery of the cells, X 700. Figs 25, 26. Tetrad and young microspores, respectively showing a strong deposition of AA. Note the intensity of staining along the walls of the microspores and tapetum, X 700.



the anther which show a high DNA intensity might contain low levels of basic proteins.

RNA and protein synthesis in the meiocytes and their derivatives appears to be low when compared to that in the sporogenous tissue. Autoradiographic studies on *Lilium* (Taylor, 1959) and *Paeonia* (Sauter, 1968, 1969) have supported this observation in *Triticum*. However, during meiosis a reduction of AA has not been observed in either *Triticum* or in *Coix* (Bhatt and Shah, 1976). Generally, low concentrations of histochemically located substances in the cells or tissues suggest a low metabolic rate. Therefore, it appears that low rates of metabolism in the pollen mother cells may bring changes during meiosis. Alternatively, a low rate of metabolism in meiocytes, dyads and tetrads in *Triticum* and in some members of the Gramineae (Rudramuniyappa, 1973) may be due to the deposition of PAS-positive substance (callose) around them (Panchaksharappa and Rudramuniyappa, 1972), a universally occurring feature in all the plants investigated. This conclusion may be supported by the observation that at the pre-meiotic stage, the sporogenous tissues and at the post-meiotic stage, the pollen cells which are undergoing mitotic divisions show no such deposition of PAS-positive substances, hence they are rich in RNA, proteins and AA. It is possible that the dividing cells may be constantly utilizing and/or exporting materials with a consequent reduction in the concentration of histochemical substances. However, lack of synthesis of these substances at this juncture may also bring about a reduction in metabolism.

Male gametophyte: Next in importance to meiosis is the differentiation of pollen. The differentiation of the male gametophyte starts in the tetrad itself at the time the latter is undergoing a deposition of PAS-positive material. In *Triticum*, the spores in the tetrad show low concentrations of RNA, proteins and DNA. In most of the plants there is little or no RNA synthesis during the early stages of microspore development (Heslop-Harrison, 1972). However, as the microspore approaches mitosis, metabolism increases, as indicated by increased levels of RNA and proteins.

The AA grains are large and deposited at the periphery of the cytoplasm of the tetrads of spores. The presence of AA stimulates enzymes namely, amylase, catalase, lipase, protease and RNAase (Saxena *et al.*, 1969). It appears that the enzymes concerned with carbohydrate metabolism may be involved in the breakdown of PAS-positive depositions, i.e., the polysaccharide material present around the tetrad of spores. The large AA grains of the tetrad have been degraded into smaller ones in the microspores and their distribution is still at the periphery. The peripheral distribution of AA in the tetrad and young microspores also seems to indicate that this substance plays a role in pollen wall formation.

In *Tradescantia* (LaCour, 1949) and *Paeonia* (Sauter, 1971), the vegetative cell is extremely active in synthesizing RNA and proteins, whereas the generative cell is inactive. Furthermore, in *Paeonia* the DNA-associated histones are higher in the generative nucleus than in the vegetative one. According to Heslop-Harrison

(1972), differences in RNA in vegetative and generative cells depends mainly on the density of ribosomes present. In *Triticum*, both the cells are active in synthesizing RNA, DNA, AA and even in 3-celled pollen they are rich in proteins and DNA. However, the DNA stainability of the vegetative nucleus of 3-celled pollen is low. The reason for such a low DNA content is not known. The mature pollen grains of the Gramineae are nutritionally independent, as they store abundant starch and lipids in addition to proteins, RNA and AA (Rudramuniyappa, 1973).

Tapetum: Much has been said regarding the nutritive role of this tissue, but no definite conclusions have yet been offered. In *Triticum*, the tapetum is secretory, being rich in RNA, DNA, AA and proteins whereas the pollen mother cells themselves contain low concentrations of these substances. Many studies have revealed the presence of high levels of DNA, RNA, proteins, AA and lipids (see Heslop-Harrison, 1972; Panchaksharappa and Rudramuniyappa, 1974, 1975; Rudramuniyappa, 1977) as well as submicroscopic organelles viz., ribosomes, amyloplasts and Golgi bodies (Marquardt *et al.*, 1968; Vasil and Aldrich, 1971; Echlin, 1971) in the tapetum. The presence of PAS-positive grains (not starch) has been recorded in the tapetum of maize and *Paspalum* (Panchaksharappa and Rudramuniyappa, 1974, 1975b). Its role in the anther is still open to question, as both sporogenous tissue and tapetum lack vascular connections and they appear side by side in the anther locule. Although many histochemical and electron microscopic studies on the tapetum reveal its metabolic and secretory nature, it is not known how these biochemical substances are secreted by this tissue. According to Heslop-Harrison (1972), the tapetum must function at least in a transmitting role in view of its location and the fact that it provides the only channel through which substances can reach the meiocytes, but there is no unequivocal evidence of transfer of these secreted products. Therefore, more direct evidence is required of its nutritional and/or translocation role.

Anther wall layer: Morphologically, the anther wall layer in the Gramineae consists of an outermost epidermis, an endothelial layer and a single middle layer. The latter disintegrates during meiosis. However, these layers exhibit very conspicuous histochemical changes (Heslop-Harrison, 1964; Milyaeva and Tsinger, 1968; Panchaksharappa and Rudramuniyappa, 1972, 1974, 1975a, b; Rudramuniyappa, 1977). It is believed that the various metabolites stored in the anther wall layers are utilised by the developing pollen (Rudramuniyappa, 1977). In the anther, RNA and proteins decline gradually as the successive wall layers differentiate, indicating that these substances are utilized during their early differentiation. At later stages of anther growth, when cell division has ceased, RNA and protein levels decrease, whereas DNA persists at a relatively high level. The anther wall layers play an important role in the nutrition of the anther, as indicated by the universal occurrence of plasmodesmata between these layers, the tapetum and

sporo-genous tissue. However, at some stage of anther growth these connections become severed between the tapetal cells and pollen mother cells, whereas they persist between the latter (see Heslop-Harrison, 1972). Therefore, it is quite possible that the increase in the nutritive substances in the tapetum may be due to the contribution of anther wall layers by way of translocation through the plasmodesmata.

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